EX. E

sequenced following amplification from the DNA of ten CD patients and two unaffected individuals. Of 35 identified SNPs, SNP1–13 selected for their rare-allele frequencies greater than 0.06, were typed in 1,272 members of the 235 CD families. SNP 12 and 13 were further identified by sequencing the 11 exons of the candidate IBD1 gene in 50 CD patients (SNP 1–13; GenBank accession numbers G67943–G67955, are submitted to dbSNP of the National Center for Biotechnology Information) and typed on the same group of individuals. To search for rare variant alleles, we subsequently investigated the 11 exons of 457 CD patients, 159 ulcerative colitis patients, and 103 unaffected unrelated individuals. All variant alleles were confirmed by sequencing a second independent amplification product.

Data analysis

Genotypic data were analysed for linkage using the NPL score of GeneHunter v2.0. Data from linkage disequilibrium mapping of CD were analysed initially with the transmission disequilibrium test[7] using a single trio (one affected and both parents) per family. Subsequently, the pedigree disequilibrium test was performed using the PDT 2.11 program[8] to analyse data from all family relatives. We estimated allele frequencies for 3 groups, 418 unrelated CD patients, 159 ulcerative colitis patients and 103 controls (including 78 unaffected, unrelated spouses of CD patients and 25 unrelated CEPH family members).

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1. Calkins, B. M. & Mendelhoff, A. I. in *Inflammatory Bowel Disease* (eds Kirsner, J. B. & Shorter, R. G.) 31–68 (Williams & Wilkins, Baltimore, 1995).
2. Hugot, J. P., Zouali, H., Lesage, S. & Thomas, G. Etiology of the inflammatory bowel diseases. *Int. J. Colorectal Dis.* 14, 2–9 (1999).
3. Hugot, J. P. *et al.* Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379, 821–823 (1996).
4. Olavesen, M. G. *et al.* Analysis of single-nucleotide polymorphisms in the interleukin-4 receptor gene for association with inflammatory bowel disease. *Immunogenetics* 51, 1–7 (2000).
5. Hugot, J. P. *et al.* Mutation screening in the CD19 and CD43 (sialophorin) genes in Crohn Disease patients. *Gastroenterology* 116, A740 (1999).
6. Zouali, H. *et al.* Refined mapping of the inflammatory bowel disease 1 gene. *Eur. J. Hum. Genet.* (submitted).
7. Spielman, R. S., McGinnis, R. E. & Ewens, W. J. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am. J. Hum. Genet.* 52, 506–516 (1993).
8. Martin, E. R., Monks, S. A., Warren, L. L. & Kaplan, N. A test for linkage and association in general pedigrees: the pedigree disequilibrium test. *Am. J. Hum. Genet.* 67, 146–154 (2000).
9. Kuster, W., Pascoe, L., Purrmann, J., Funk, S. & Majewski, F. The genetics of Crohn's disease: complex segregation analysis of a family study with 265 patients with Crohn's disease and 5387 relatives. *Am. J. Med. Genet.* 32, 105–108 (1989).
10. Monsen, U., Iselius, L. & Hellers, G. Evidence for a recessive gene in Crohn's disease. *Acta Chir. Scand.* 559 (suppl.), 7–42 (1990).
11. Orholm, M. *et al.* Investigation of inheritance of chronic inflammatory bowel disease by complex segregation analysis. *Br. Med. J.* 306, 20–24 (1993).
12. Colombel, J. F. *et al.* Clinical characteristics of Crohn's disease in 72 families. *Gastroenterology* 111, 604–607 (1996).
13. The IBD International Genetics Consortium. International collaboration provides convincing linkage replication in complex disease through analysis of a large pooled data set: Crohn disease and chromosome 16. *Am. J. Hum. Genet.* 68, 1165–1171 (2001).
14. Ogura, Y. *et al.* Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB. *J. Biol. Chem.* 276, 4812–4818 (2001).
15. Inohara, N., Ogura, Y., Chen, F. F., Muto, A. & Nuñez, G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J. Biol. Chem.* 276, 2551–2554 (2001).
16. Inohara, N. *et al.* Nod 1, an Apaf-1-like activator of caspase-9 and nuclear factor κB. *J. Biol. Chem.* 274, 14560–14567 (1999).
17. Bertin, J. *et al.* Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-κB. *J. Biol. Chem.* 274, 12955–12958 (1999).
18. Inohara, N. *et al.* An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* 275, 27823–27831 (2000).
19. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–2088 (1998).
20. Sundberg, J. P., Elson, C. O., Bedigian, H. & Birkenmeier, E. H. Spontaneous, heritable colitis in a new substrain of C3H/HeJ mice. *Gastroenterology* 107, 1726–1735 (1994).
21. McKay, D. M. Intestinal inflammation and the gut microflora. *Can. J. Gastroenterol.* 13, 509–516 (1999).
22. Schreiber, S., Nikolaus, S. & Hampe, J. Activation of nuclear factor κB in inflammatory bowel disease. *Gut* 42, 477–484 (1998).
23. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. & Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. *Science* 270, 286–290 (1995).
24. Wahl, C., Liptay, S., Adler, G. & Schmid, R. M. Sulfasalazine: a potent and specific inhibitor of nuclear factor κB. *J. Clin. Invest.* 101, 1163–1174 (1998).
25. Satsangi, J. *et al.* Two stage genome wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosome 3, 7 and 12. *Nature Genet.* 14, 199–202 (1996).
26. Hampe, J. *et al.* Linkage of inflammatory bowel disease to Human chromosome 6p. *Am. J. Hum. Genet.* 65, 1647–1655 (1999).
27. Cho, J. H. *et al.* Linkage and linkage disequilibrium in chromosome band 1p36 in American Chaldeans with inflammatory bowel disease. *Hum. Mol. Genet.* 9, 1425–1432 (2000).
28. Duerr, R. H., Barmada, M. M., Zhang, L., Pfützer, R. & Weeks, D. E. High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. *Am. J. Hum. Genet.* 66, 1857–1862 (2000).
29. Rioux, J. D. *et al.* Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am. J. Hum. Genet.* 66, 1863–1870 (2000).
30. Lennard-Jones, J. E. Classification of inflammatory bowel disease. *Scand. J. Gastroenterol.* 170 (Suppl.), 2–6 (1989).

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A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease

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Crohn's disease is a chronic inflammatory disorder of the gastro-intestinal tract, which is thought to result from the effect of environmental factors in a genetically predisposed host. A gene location in the pericentromeric region of chromosome 16, *IBD1*, that contributes to susceptibility to Crohn's disease has been established through multiple linkage studies[1–6], but the specific gene(s) has not been identified. *NOD2*, a gene that encodes a protein with homology to plant disease resistance gene products is located in the peak region of linkage on chromosome 16 (ref. 7). Here we show, by using the transmission disequilibium test and case-control analysis, that a frameshift mutation caused by a cytosine insertion, 3020insC, which is expected to encode a truncated NOD2 protein, is associated with Crohn's disease. Wild-type NOD2 activates nuclear factor NF-κB, making it responsive to bacterial lipopolysaccharides; however, this induction was deficient in mutant NOD2. These results implicate *NOD2* in susceptibility to Crohn's disease, and suggest a link between an

innate immune response to bacterial components and development of disease.

The idiopathic inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis, are chronic disorders of the gastrointestinal tract with unknown aetiology, and with a combined prevalence of about 150–200 cases per 100,000 in western countries[8,9]. Although the aetiology of IBD is unknown, an abnormal inflammatory response directed against enteric microflora in a genetically susceptible host has been proposed[10]. Familial clustering of disease and studies of twins strongly suggest that IBD, and in particular CD, is a genetic disorder[11]. Genome-wide searches for IBD-susceptibility genes have resulted in the identification of several loci for CD and/or ulcerative colitis, most notably for CD, in the pericentromeric region of chromosome 16 (*IBD1*)[1–6].

NOD2 has structural homology with both the apoptosis regulators Apaf-1/Ced-4 and a class of plant disease resistant (R) gene products[7]. Like the latter gene products, NOD2 comprises an amino-terminal effector domain, a nucleotide-binding domain and leucine-rich repeats (LRRs) (ref. 7). *NOD2* has been mapped to chromosome 16q12 (ref. 7) and is tightly linked to markers D16S3396, D16S416 and D16S419 (Fig. 1a)—a site that precisely overlaps with *IBD1* (ref. 1). Given the genomic localization and the role of NOD proteins in recognizing bacterial components[12], we thought that *NOD2* might function as a susceptibility gene for CD.

The 12-exon genomic organization of the *NOD2* gene was determined by aligning the complementary DNA sequence (AF178930) with our genomic bacterial artificial chromosome (BAC) clone RP11-327F22 (AC007728) (Fig. 1a). All coding exons and flanking introns were sequenced in 17 affected individuals from pure CD families with increased linkage scores at D16S3396, as well as in 4 case controls. In three CD patients, a cytosine insertion was observed in exon 11 at nucleotide 3020 (3020insC) (Fig. 1b). 3020insC resulted in a frameshift at the second nucleotide of codon 1007 (Fig. 1b), and a Leu1007→Pro substitution in the tenth LRR, followed by a premature stop codon (Fig. 1c). The predicted truncated NOD2 protein contained 1,007 amino acids instead of the 1,040 amino acids of the wild-type NOD2 protein (Fig. 1d).

We used an allele-specific polymerase chain reaction (PCR) assay (Fig. 2) to type 3020insC in IBD families and case controls. Analysing only one CD patient per independent family, we observed preferential transmission (Table 1) from heterozygous parents to affected children of 3020insC (39 transmissions and 17 non-transmissions; $P = 0.0046$). Analysing all independent nuclear families by sib-TDT (ftp://lahmed.stanford.edu/pub/aspex/index.html), the empirical P value was similar, $P = 0.0007$. As expected from linkage studies[2,3], no preferential transmission of 3020insC was observed among families with ulcerative colitis (data not shown). As 365 of the 416 independent CD families have several affected individuals, the applicability of these associations to the more common, sporadic cases requires further study.

Additional support for association to CD was provided by case-

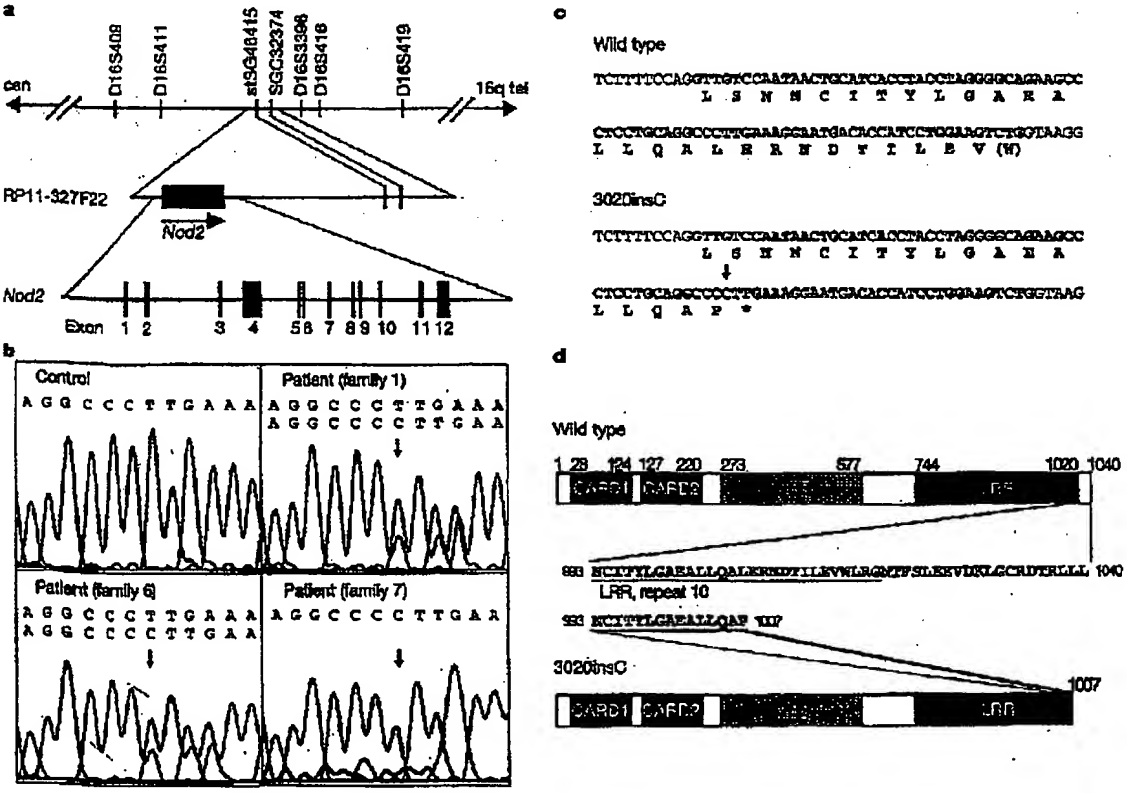

Figure 1 Identification of a frameshift *NOD2* mutation in affected individuals from CD families. a, Physical map of the region of interest at 16q12. Approximate positions of chromosomal and genetic markers are based on ref. 22. The human genomic BAC clone RP11-32722 contains the *NOD2* gene and markers stSG46415 and SGC32374. The exon–intron organization of the human *NOD2* gene is shown underneath. b, DNA sequence electropherograms (exon 11) from control and three affected individuals from [illegible]. Patients from families 1 and 6 are heterozygous, whereas the patient from [illegible] homozygous for 3020insC. The cytosine insertion is indicated by an arrow. c, Nucleotide and predicted amino-acid sequence of exon 11 and flanking introns from wild-type control and patients with 3020Cins. The exon sequence is shown in bold. The site of 3020insC is indicated by an arrow. Residue (W) indicates that a nucleotide from exon 12 contributes to the codon. d, Domain structure of NOD2, illustrating the site of protein truncation. Caspase-recruitment domains (CARDs), the nucleotide-binding domain (NBD) and ten LRRs are shown. Residues of the tenth LRR are underlined. Numbers indicate residue positions.

Table 1 TDT demonstrates preferential transmission of the 3020insC to CD patients

	One CD patient per family			All CD patients	
Source	Transmitted	Not transmitted	P value	Transmitted	Not transmitted
Univ. of Chicago	21	10		32	16
Johns Hopkins	4	4		10	8
Univ. of Pittsburgh	14	3		26	9
Total	39	17	0.0046	68	33

Table 2 Allele frequency of 3020insC in unrelated Crohn's disease patients and controls

	Crohn's disease			Case controls	
Source	Sample size	3020insC*	Source	Sample size	3020insC*
Univ. of Chicago	212	7.3	Chicago	65	3.8
Johns Hopkins	68	6.6	Baltimore	46	3.2
Univ. of Pittsburgh	116	10.8	San Francisco	81	3.1
			Germany	94	5.3
Total	416	8.2		287	4.0

For analysis, one CD patient was selected from 416 independent families, the difference in allele frequency between CD patient and control was significant ($P = 0.0018$, by the large sample approximations to a two sample binomial test).
*Per cent allele frequency.

control analysis, in which, using one CD individual per independent family, the 3020insC allele frequency among all CD groups was 8.2% (Table 2). The allele frequencies of 3020insC were comparable among Jewish (8.4%) and non-Jewish Caucasians (8.1%). Among case controls (Table 2), the allele frequency in four separate Caucasian cohorts of 4.0% was significantly lower than in CD patients ($P = 0.0018$, by large-sample approximations to a two-sample binomial test). The allele frequency of the 3020insC among 182 unrelated ulcerative colitis patients was 3.0%, and was significantly lower than the frequency among CD patients ($P = 0.0010$). The genotype frequencies of 3020insC in unrelated CD individuals were 11 homozygotes, 46 heterozygotes and 359 wild-type homozygotes.

Among case controls, there were 23 heterozygous individuals, with the remaining being wild-type homozygotes. The genotype-relative risk (GRR) for heterozygous and homozygous 3020insC was 1.5 and 17.6, respectively, as compared with wild-type controls. Given its frequency, 3020insC is unlikely to account completely for the observed evidence of linkage at *IBD1*, and other variants of *NOD2* may confer additional disease risk. For example, two single-nucleotide polymorphisms in *NOD2* have been identified, 2722G→C (Gly908Arg) and 2104C→T (Arg702Trp), which are highly associated with CD by the transmission disequilibrium test (data not shown). Furthermore, other susceptibility genes might also be present in this broad region[1–6] of linkage on chromosome 16.

NOD2 has been shown to activate NF-κB and to confer responsiveness to bacterial lipopolysaccharides[7,12]. To test the ability of wild-type and mutant NOD2 to activate NF-κB, human embryonic kidney (HEK) 293T cells were transiently co-transfected with wild-type or 3020insC plasmids and an NF-κB reporter construct. In the absence of lipopolysaccharide (LPS), expression of both wild-type and mutant NOD2 induced activation of NF-κB (Fig. 3a). Notably, equivalent levels of wild-type and mutant NOD2 protein expression (as assessed by immunoblotting of total lysates) resulted in similar levels of NF-κB activation (Fig. 3a).

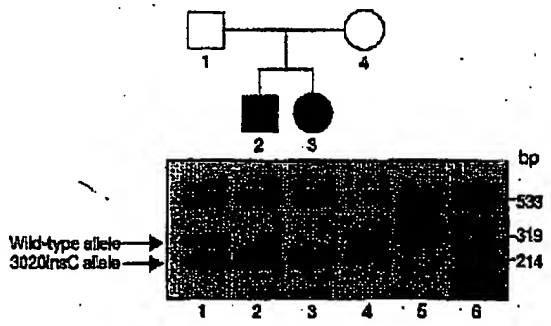

Figure 2 Determination of transmission of the 3020insC mutation in a CD family by allele-specific PCR. Multiplex PCR was used to generate a nonspecific 533-bp product, along with allele-specific amplicons: a 319-bp fragment (wild type) and a 214-bp fragment (3020insC). In this family, both parents (lanes 1 and 4) are heterozygous for 3020insC, whereas both children (lanes 2 and 3) have CD and are homozygous for 3020insC. Lane 5, wild-type control; lane 6, pBR322 DNA MspI markers. Numbers on the right indicate the size of fragments.

Like NOD2, cytosolic plant disease resistant proteins have carboxy-terminal LRRs that are critical for the recognition of pathogen components and induction of pathogen-specific responses[13–15]. We therefore compared the ability of wild-type and mutant NOD2 proteins to induce NF-κB activity in response to LPS. Because overexpression of NOD2 induces potent NF-κB activation (Fig. 3a), we transfected the cells with low amounts of wild-type and mutant

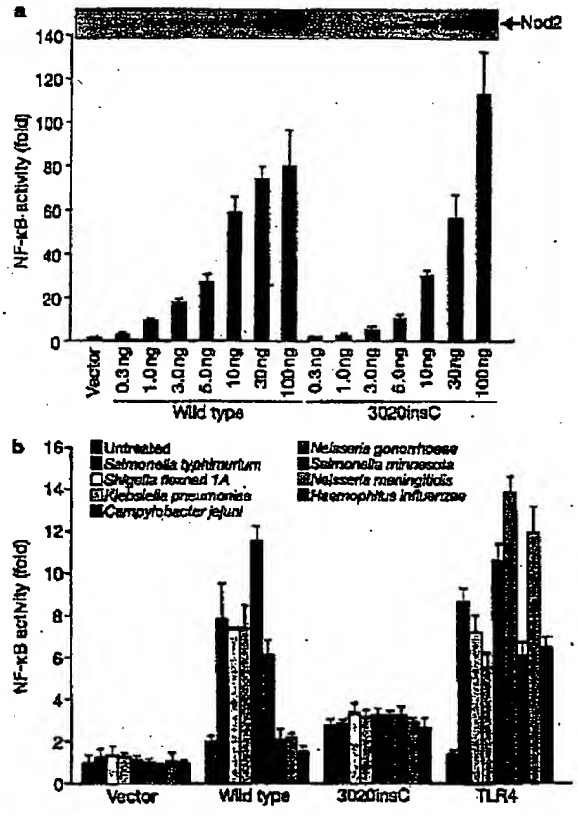

Figure 3 Differential responsiveness of wild-type and mutant NOD2 to LPS. a, HEK293T cells were co-transfected in triplicate with the indicated amounts of pcDNA3 (vector), wild-type pcDNA3-NOD2, or pcDNA3-NOD2 3020insC and pEF-BOS-β-gal and pBVI-luc reporter plasmids. Values represent means ± s.d. Expression of wild-type and mutant NOD2 proteins in cell extracts is shown on top. b, HEK293T cells were co-transfected in triplicate with 0.3 ng of pcDNA3-NOD2, 3 ng of pcDNA3-NOD2 3020insC, 3 ng of pcDNA3-TLR4 plus 3 ng of pcDNA3-MD-2 (indicated by TLR4) or pcDNA3 (vector) and pEF-BOS-β-gal and pBOV-luc. Under these conditions, both wild-type and mutant NOD2 constructs induced similar levels of basal NF-κB activity. Eight hours after transfection, cells were treated with 10 μg ml^{-1} of LPS from indicated bacteria. Values represent means ± s.d. Results are representative of at least five independent experiments.

NOD2 plasmids to induce similar levels of protein expression and basal NF-κB activity (Fig. 3a). LPS from various bacteria induced NF-κB activation in cells expressing wild-type NOD2, but not in cells transfected with control plasmid (Fig. 3b).

Significantly, the ability of mutant NOD2 to confer responsiveness to LPS was greatly diminished when compared with wild-type NOD2 (Fig. 3b). Differential regulation of NOD2 function by LPS from different bacteria was observed (Fig. 3b), whereas all LPS preparations induced NF-κB activation comparably in cells transfected with Toll-like receptor-4 (TLR-4), a cell-surface receptor for LPS[16].

The innate immune system regulates the immediate response to microbial pathogens and is initiated by recognition of specific pathogen components by receptors in host immune cells[16]. NOD1 and NOD2 seem to function as intracellular receptors for LPS with the LRRs required for responsiveness[12]. We have shown here that truncation of the tenth LRR of NOD2 is associated with CD. Consistent with earlier linkage studies[2,5], this variant is associated solely with CD, and not with ulcerative colitis. Functional analyses indicate that the disease-associated NOD2 variant is significantly less active than the wild-type protein in conferring responsiveness to bacterial LPS. In plant NOD2 homologues, the LRRs determine the specificity for pathogen products and alterations in LRRs can result in unresponsiveness to particular pathogens[13–15]. Similarly, genetic variation in the LRRs of TLR4 account for inter-individual differences in bronchial responsiveness to aerosolized LPS[17].

Several mechanisms can be envisaged to account for susceptibility to CD in individuals carrying this variant. NOD2 is a cytosolic protein whose expression is restricted to monocytes, with no expression detected in lymphocytes[7]. A deficit in sensing bacteria in monocytes/macrophages might result in an exaggerated inflammatory response by the adaptive immune system. A related possibility is that wild-type NOD2 may mediate the induction of cytokines such as interleukin-10 that can downregulate the inflammatory response[18,19]. Finally, variation in the LRRs of plant NOD2 homologues can result in recognition of new specificities for pathogen components[13,14]. Thus, it is also possible that NOD2 variants might act as gain-of-function alleles for unknown pathogens. Our studies implicate *NOD2* in susceptibility to CD, and suggest a link between an innate response to bacterial components and development of disease. □

Methods

IBD families

IBD families were ascertained for linkage and association studies (affected child with both parents) through the University of Chicago, the Johns Hopkins Hospital and the University of Pittsburgh. In all cases informed consent for a molecular genetic study was obtained, and the study protocol was approved by the individual institutional review boards.

Allele-specific PCR

We used primers framing a 539-base-pair region surrounding the 3020insC allele to amplify genomic DNA isolated from controls and patients by PCR (sense, 5′-CTGAGCCTTTGTTGATGAGC-3′; antisense, 5′-TCTTCAACCACATCCCCATT-3′). In addition, each PCR reaction contained two additional primers designed to detect the wild-type allele (sense, 5′-CAGAAGCCCTCCTGCAGGCCCT-3′) and another primer designed to detect the 3020insC allele (antisense, 5′-CGCGTGTCATTCCTTTCAT GGGGC-3′). The 3020insC was confirmed by DNA sequencing. We performed multiplex PCR with all four primers in one tube. PCR products were isolated on 2% agarose gels and visualized with ethidium bromide.

Data analysis

The *P* values for the TDT test[20] were calculated using a binomial exact test. Simulations (500,000 replicates) were done using the sib-TDT software (ftp://ftp.hmed.stanford.edu/pub/asper/index.html) to calculate empirical probabilities for the TDT χ^2 statistic when all independent nuclear families were counted. This calculation was done by permuting parent alleles while fixing the IBD status of siblings within a family. We estimated the frequency of the genotypes in the affected individuals from 416 unrelated CD patients. The [illegible] is determined as the ratio of the marginal penetrance of the 3020insC homozygote [illegible] to the wild-type homozygotes. Using Bayes rule, the GRRs can [illegible] and control groups. For the [illegible] equilibrium.

Expression plasmids and immunoblotting

The expression plasmids pcDNA3-NOD2, pcDNA3-TLR4 and pcDNA3-MD-2 have been described[7,12]. The expression plasmid producing the NOD2A33 mutant (3020insC) was generated by PCR and cloned into pcDNA3-(Invitrogen), and confirmed by DNA sequencing. Expression of untagged NOD2 proteins in transfected cells was determined by immunoblotting using affinity-purified rabbit anti-NOD2 antibody, as described[7]. To raise this antibody, we overexpressed recombinant NOD2 protein (residues 28–301) in *Escherichia coli* strain BL21(DE3) using the pET-30a vector (Novagen). Recombinant NOD2 protein containing a C-terminal histidine tag was purified using a nickel column (Novagen) and injected into rabbits.

NF-κB activation assay

We carried out NF-κB activation assays as described[7,12]. Briefly, HEK293T cells were co-transfected with 12 ng of the reporter construct pBVI-Luc, the indicated amounts of each expression plasmid and 120 ng of pEF-BOS-β-gal in triplicate in the presence or absence of LPS[7,12]. LPS from various sources were obtained from Sigma or from several investigators. Twenty-four hours after transfection, cell extracts were prepared and the relative luciferase activity was measured as described[7,12]. Results were normalized for transfection efficiency with values obtained with pEF-BOS-β-gal.

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1. Hugot, J. P. *et al.* Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 379, 821–823 (1996).
2. Ohmen, J. D. *et al.* Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohn's disease, but not in ulcerative colitis. *Hum. Mol. Genet.* 5, 1679–1683 (1996).
3. Cuthbert, M. B. *et al.* Genetic analysis of inflammatory bowel disease in a large European cohort supports linkage to chromosomes 12 and 16. *Gastroenterology* 115, 1066–1071 (1998).
4. Cavanaugh, J. A. *et al.* Analysis of Australian Crohn's disease pedigrees refines the localization for susceptibility to inflammatory bowel disease on chromosome 16. *Ann. Hum. Genet.* 62, 291–298 (1998).
5. Cho, J. H. *et al.* Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc. Natl Acad. Sci. USA* 95, 7502–7507 (1998).
6. Annese, V. *et al.* Genetic analysis in Italian families with inflammatory bowel disease supports linkage to the IBD1 locus—a GISC study. *Eur. J. Hum. Genet.* 7, 567–573 (1999).
7. Ogura, Y., Inohara, N., Benito, A., Chen, F. F. & Nuñez, G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB. *J. Biol. Chem.* 276, 4812–4818 (2001).
8. Loftus, E. V. Jr *et al.* Crohn's disease in Olmsted County, Minnesota, 1940–1993: incidence, prevalence, and survival. *Gastroenterology* 114, 1161–1168 (1998).
9. Kyle, J. Crohn's disease in the northeastern and northern Isles of Scotland: an epidemiological review. *Gastroenterology* 103, 392–399 (1992).
10. Fiocchi, C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 115, 182–205 (1998).
11. Binder, V. Genetic epidemiology of inflammatory bowel disease. *Digest. Dis.* 16, 351–355 (1998).
12. Inohara, N., Ogura, Y., Chen, F. F., Muto, A. & Nuñez, G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J. Biol. Chem.* 276, 2551–2554 (2001).
13. Parniske, M. *et al.* Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the Cf-4/9 locus of tomato. *Cell* 91, 821–832 (1997).
14. Ellis, J. G., Lawrence, G. J., Luck, J. E. & Dodds, P. N. Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. *Plant Cell* 11, 495–506 (1999).
15. Dixon, M. S., Golstein, C., Thomas, C. M., van Der Biezen, E. A. & Jones, J. D. Genetic complexity of pathogen perception by plants: the example of Rcr3, a tomato gene required specifically by Cf-2. *Proc. Natl Acad. Sci. USA* 97, 8807–8814 (2000).
16. Aderem, A. & Ulevitch, R. Toll-like receptors in the induction of the innate immune response. *Nature* 406, 782–787 (2000).
17. Arbour, N. C. *et al.* TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genet.* 25, 187–191 (2000).
18. Moore, K. W. *et al.* Interleukin-10. *Annu. Rev. Immunol.* 11, 165–190 (1993).
19. Berg, D. J. *et al.* Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4+ TH1-like responses. *J. Clin. Invest.* 98, 1010–1020 (1996).
20. Spielman, R. S. & Ewens, W. J. The TDT and other family-based tests for linkage disequilibrium and association. *Am. J. Hum. Genet.* 59, 983–989 (1996).
21. Risch, N. & Merikangas, K. The future of genetic studies of complex human diseases. *Science* 273, 1516–1517 (1996).
22. Han, C. S. *et al.* Construction of a BAC contig map of chromosome 16q by two-dimensional overgo hybridization. *Genome Res.* 10, 714–721 (2000).

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